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# Changes in Protein Contents and Activities of Proteolytic Enzymes in *Medicago sativa* During Regrowth

Kim, Tae-Hwan* and Byung-Ho Kim[1]

*Institute for Development of Livestock Production; and*

[1]*Department of Dairy Science, Gyeongsang National University, Chinju 660-701, Korea*

**An experiment with non-nodulating alfalfa (*Medicago sativa* L.) plants was designed to investigate the changes in protein contents and the activities of proteolytic enzymes during a regrowth period of 24 d. Shoot removal caused a depression of root growth and significantly reduced protein contents in roots. An initial decline of root proteins for the first 10 d was followed by a rapid recovery from d 11 to 24. The major increase of regrowing shoot weight occurred also from d 11. The activities of aminopeptidase and endoprotease slightly decreased in regrowing leaves, while protein contents remains stable after shoot removal. Roots exhibited source behaviour with a rapid increase of endoprotease activities for the first 10 d of regrowth; about a 370% increase over the initial level was observed. Increase in endoprotease activity in roots coincided with the time of protein remobilization after shoot removal, indicating the important role of endoproteases in protein degradation.**

*Keywords* : *Medicago sativa* L., regrowth, soluble protein, proteolytic activity

In perennial forage legumes periodically defoliated by cutting or grazing, nitrogen cycling following defoliation can be affected to optimize nitrogen supply for earlier shoot regrowth. Shoot regrowth following defoliation is a complex process in which the action and interaction of many factors determine ultimate shoot yield. It is now well known that organic reserves from roots and stubble contribute to regrowth of defoliated or grazed plant. The classic discussion of this concept emphasized the importance of carbohydrate reserves in the roots, which are greater pool size and show a greater decrease after shoot removal (Hodgkinson, 1969; Rappport and Travis, 1984). During the last decade, the experiments with isotopically labelled nitrogen have provided evidence for the turnover of reduced nitrogen compounds in roots and stubble during shoot regrowth of *Bromus mollis* (Phillips *et al.*, 1983), of *Lolium perenne* (Ourry *et al.*, 1988) and of *Medicago sativa* (Kim *et al.*, 1991, 1993). These remobilization of nitrogen reserves occurred mainly during early regrowth and accounted for nearly all nitrogen of regrowing shoots for 6 d after defoliation in *L. perenne* (Ourry *et al.*, 1989) and for 10 d in *M. sativa* (Kim *et al.*, 1991).

A number of results from the analyses of principal nitrogenous compounds have demonstrated that proteins constitute the major source of nitrogen reserves in remaining organs after defoliation in *M. sativa* (Constable *et al.*, 1977; Hendershot and Volenec, 1993). In *L. perenne* soluble proteins in roots and stubble decreased to respectively 45 and 40% during the first 4 d after defoliation, while those in regrowing leaves gradually increased (Ourry *et al.*, 1989). These authors estimated that 70% of protein in regrowing leaves were derived from protein remobilization during the first 6 d of regrowth. Bradly and Volenec (1992) also observed a decline of protein content to 65% of the initial level in taproots of *M. sativa* during 28 d of regrowth.

Regrowth after defoliation in *L. perenne* was accompanied by a rapid increase in free amino acids

*Corresponding author: Fax +82-591-751-6113

in leaves and a progressive decrease in roots (Lefevre *et al.*, 1991), and a high Asn/Gln ratio in the xylem sap during the first 4 d of regrowth (Bigot *et al.*, 1991). The latter authors suggest that Asn and Gln produced in roots and stubble are rapidly translocated to regrowing leaves, and they act as a temporary storage of soluble organic nitrogen when nitrate reduction is very low during the early regrowth period. In regrowing *M. sativa*, Kim and Boucaud (1993) also observed that the relative content of Asn, which was the main transported form of reduced nitrogen in roots, decreased during the first 10 d of regrowth. Remobilization of protein-N from roots and stubble are clearly associated with the activity of proteolytic enzymes, changes in their levels after defoliation have not yet well described.

Due to the obvious diminution of protein pool in roots and stubble after defoliation and the evidence of remobilization of nitrogen reserves to regrowing shoots, we have focused on enzymes related to protein catabolism. The objectives of this study were 1) to investigate the effect of defoliation on protein contents in roots and regrowing leaves, and 2) to determine the activities of key enzymes involved in proteolysis during the regrowth period.

## MATERIALS AND METHODS

### Growth condition and experimental procedure

Alfalfa (*Medicago sativa* L. cv. Europe) seeds were sterilized and germinated in a sand bench. When primary trifoliate leaves were developed, 15 seedlings per 9 L culture pot were transplanted and grown hydroponically on a continuously aerated nutrient solution as previously described by Kim *et al.* (1991). Plants were grown in a growth cabinet with a 16/8 h of light/dark cycle and with a 23/18℃ of thermoperiod. Relative humidity maintained at 70%.

Plants were grown until the early flowering stage (about 10-wk-old), thereafter shoots were cut to a height of 6 cm above taproot level. They were then allowed to regrow and the second cutting was carried out on the 24th d of regrowth. Fresh samples of roots and leaves were collected on 0, 1, 2, 3, 4, 6, 10, 14 and 24 d after the second cutting. Values given in this paper, therefore, always refer to this second regrowth cycle.

### Preparation of cell-free extracts

Six grams of fresh tissues were ground in a cold mortar and pestle with 24 mL of extraction buffer consisting of 100 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 10 mM mercaptoethanol. The extracts were filtered through two layers of cheesecloth and centrifuged at 25,000 *g* for 30 min. The volume of the supernatant were adjusted to 25 mL with the appropriate buffer. The aliquots were desalted on Sephadex G25. This protein fraction was assayed for the determination of soluble proteins and used as the enzyme source. Soluble proteins were quantitated using the method described by Lowry *et al.* (1951). All steps of extraction procedure were carried out at 4℃. For each enzyme assay, 3 replicates of enzyme extraction and the corresponding activity measurements were performed.

### Enzyme assays

Aminopetidase activities were measured by using leucine *p*-nitroanilide (LPN). The reaction mixture contained 0.1 mL of desalted extract and 1.9 mL of 50 mM Tris-HCl buffer (pH 7.5) containing 2 mM LPN, 1 mM EDTA, and 5 mM mercaptoethanol. After 2 h at 40℃ the reaction was stopped by adding 0.5 mL of 30% (w/v) acetic acid. The precipitated protein was removed by centrifugation at 2000 *g* for 10 min and the $A_{410}$ of the supernatant was read. Activity is expressed as $\Delta A_{410} \cdot \text{mg protein}^{-1} \cdot \text{h}^{-1}$.

Carboxypeptidase activities were measured with *N* carbobenzoxy-phenylalanine-alanine (N-CBZ-PHE-ALA). 0.3 mL of desalted extract was mixed with 0.7 mL of medium (50 mM citrate-phosphate buffer at pH 5) containing 2 mM N-CBZ-PHE-ALA, 1 mM EDTA, and 5 mM mercaptoethanol. The mixture was incubated at 40℃ for 2 h, and then the reaction was stopped by adding 1 mL of 10% (w/v) trichloroacetic acid (TCA). After centrifugation at 2000 *g* for 10 min, 0.1 mL of the supernatant was incubated with 1 mL of ninhydrin reagent at 100℃ for 20 min. To estimate the activity of primarily exoproteases, the TCA-soluble supernatant was assayed for ninhydrin-positive compounds. The calibration of ninhydrin was performed with leucine. Leucine released into the medium was determined. Activity

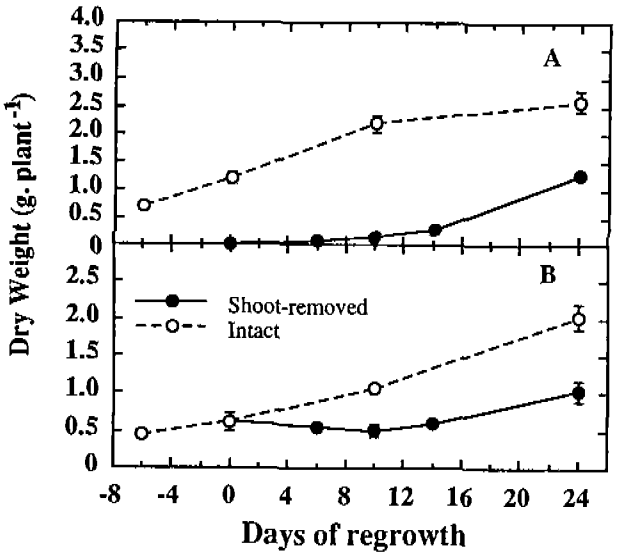


**Fig. 1.** Growth of (A) shoots and (B) roots of shoot removed and intact *Medicago sativa* L. Each value is the mean± SE of three replicates.

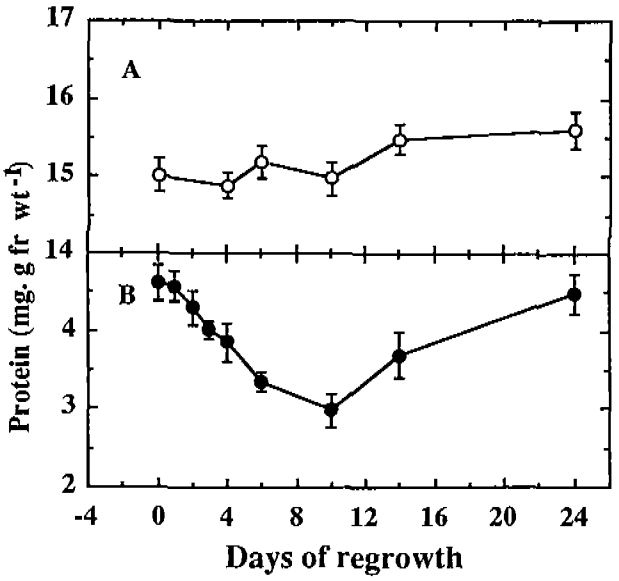


**Fig. 2.** Changes in protein concents of leaves (A) and roots (B) during a regrowth period of 24 d. The measurement of leaves at d 0 is carried out from remaining leaves after shoot removal. Each value is the mean± SE of three replicates.

is expressed as mM Leu·mg protein$^{-1}$·h$^{-1}$.

Endoprotease (azocaseinase) activities were determined at pH 5 and 7.5 as suggested in several studies (Chrispeels and Boulter, 1975; Drivdahl and Thimann, 1977). At pH 5, the reaction mixture contained 0.3 mL of desalted extract and 0.7 mL of 50 mM citrate-phosphate buffer (pH 5), containing 0.7 mg of azocasein, 1 mM EDTA, and 5 mM mercaptoethanol. After incubation at 40℃ for 2 h, reaction was stopped by adding 1 mL of 10% (w/v) TCA and by abrupt cooling. The $A_{340}$ of the supernatant was read after centrifugation at 2000*g* for 10 min. For the enzyme assay at pH 7.5, azocasein was dissolved in 50 mM Tris-HCl buffer (pH 7.5). Activity is expressed as $\Delta A_{340}$·mg protein$^{-1}$·h$^{-1}$.

## RESULTS

### Shoot and root growth

Shoots regrew slowly for 10 d after shoot removal. From 11 to 24 d after shoot removal, shoot weight increased rapidly so that total shoot weight exceeded the initial level (Fig. 1A). On the other hand, shoot weight of intact plants increased further through d 10 and then remained unchanged until d 24 (Fig. 1A). The intact plants were in full bloom by d 10. Roots of the shoot-removed plants grew little during the regrowth period. In intact plants dry matter of roots continuously increased and maintained much higher than those of shoot-removed plants throughout the course of the experiment (Fig. 1B).

### Protein contents

Protein contents in leaves did not significantly change during the first 10 d of regrowth, and they increased slightly thereafter. Comparing with data obtained from before and after shoot removal, there was not a significant difference, ranging from 14.6 to 15.6 mg·g fr wt$^{-1}$ (Fig. 2A).

Shoot removal significantly reduced protein contents in roots. Soluble proteins in roots from the shoot-removed plants decreased rapidly from d 0 to d 10, and then recovered rapidly from d 10 to d 24. The contents at d 10 was the lowest showing about 65% of the initial level at d 0 (Fig. 2B).

### Proteolytic enzyme activities

Aminopeptidase activities in regrowing leaves significantly decreased, reaching a minimum at d 14, and then stabilized at about 59% of the initial level at d 0 (Fig. 3A), while protein contents remained unchanged throughout the experiment (Fig. 2A). Aminopeptidase activities in roots increased slightly

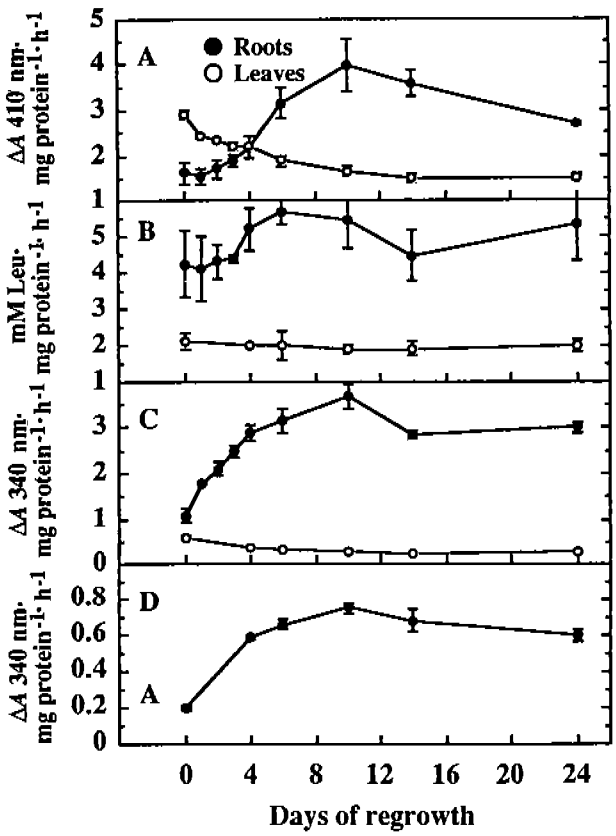


**Fig. 3.** Changes in the activities of aminopetidase (A), carboxypeptidase (B), azocaseinase at pH 5 (C) and azocaseinase at pH 7.5 (D) during a regrowth period of 24 d. The measurement of leaves at d 0 is carried out from remaining leaves after shoot removal. Each value is the mean± SE of three replicates.

for the first 4 d, and then increased rapidly from d 4 to a maximum at d 10 (a 340% increase over the initial level). From d 11 the activities decreased rapidly until d 24 (Fig. 3A). The activities of the leaves were higher during the first 4 d of regrowth, thereafter those of roots were higher.

Carboxypeptidase activities in the leaves ranged from 1.9 to 2.1 mM Leu·mg protein$^{-1}$·h$^{-1}$ throughout the experimental period (Fig. 3B). In roots, the carboxypeptidase activities slightly increased until d 10 after an initial lag for the first 3 d of regrowth. From d 10 the activities remained unchanged (Fig. 3B). Comparing with the initial value at d 0, there was no significant effect of shoot removal on the carboxypetidase activities in leaves and roots. The activities in roots remained much hugely than those in leaves throughout the entire experimental period.

Endoprotease activities, measured at pH 5, in leaves slightly decreased from 0.60 to 0.24 $\Delta A_{340}$·mg protein$^{-1}$·h$^{-1}$ during the period of regrowth as compared to the levels before shoot removal (Fig. 3C). The activities (pH 5) in roots increased rapidly from d 1 to a maximum at d 10 (an increase of 370% over the initial level). The activities decreased slightly from d 11 to d 14, then stabilized until d 24 (Fig. 3C).

Endoprotease activities at pH 7.5 from leaf tissues were too low with a considerable variance so that we did not determine them. The activities (pH 7.5) in roots followed a similar pattern to those measured at pH 5 although enzyme activities were much lower. A maximum activity at d 10 was 0.75 $\Delta A_{340}$·mg protein$^{-1}$·h$^{-1}$, which corresponded to 375% of the initial level at d 0 (Fig. 3D).

Concerning the proteolytic activities during the regrowth period, the difference between the two plant parts was clear: the activities of leaf enzymes decreased or maintained at the initial level whereas those of root enzymes increased until d 10, followed by a decrease. In addition, endopetidase in roots was much responsive to shoot removal than exopeptidase: the activities of aminopeptidase and carboxypetidase showed an initial lag for the first 3 to 4 d of regrowth whereas those of endoprotease immediately increased from the first d of regrowth.

## DISCUSSION

Shoot removal of *M. sativa* has a strong and rapid effect internal cycling of nitrogen (Groat and Vance, 1981). This work showed that shoot regrowth proceeded slowly for 10 d following defoliation and shoot removal caused a depression of root growth (Fig. 1). Similar observation in *M. sativa* for shoot and root growth following shoot removal has been reported (Crall and Heichel, 1981). These results suggest that early regrowth is interrupted by low supply of current photosynthates and nitrogen assimilates. Early regrowth following shoot removal was characterized by low assimilation of mineral nitrogen (Ourry *et al.*, 1988: Jarvis and Macduff, 1989) and from atmospheric $N_2$ (Vance *et al.*, 1979; Denison *et al.*, 1992; Kim *et al.*, 1993). Therefore, early regrowth depends upon nitrogen reserves exclusively in the roots to meet the nitrogen demands of shoot.

The data obtained in this experiment showed that shoot removal did not significantly affect the con-

tents of soluble proteins in leaves, while it caused a rapid decline in the roots until the first 10 d of regrowth (Fig. 2A). Soluble proteins in roots decreased about 36% of the initial level for 10 d of regrowth (Fig. 2B). We observed in a previous study (Kim and Boucaud, 1993) that protein-$^{15}N$ in taproot and lateral roots decreased to respectively 35.1 and 25.5% during the first 10 d after shoot removal. The changes in soluble protein contents in nodules (Groat and Vance, 1981) and in taproot (Bradly and Volenec, 1992) of regrowing *M. sativa* also showed the similar pattern. These results could be taken to indicate that shoot removal induces the active protein degradation in source organs to meet the demands for new shoot growth during the early regrowth, when exogenous nitrogen supply is limited. Seventy percent of proteins in regrowing leaves were derived from the proteins remobilized during the first 6 d of regrowth in *L. perenne* (Ourry *et al.*, 1989).

The activities of aminopeptidase and endoprotease decreased in regrowing leaves (Fig. 3A, C), while those of carboxypeptidase remained stable throughout the regrowth period (Fig. 3B). In the roots of shoot-removed plants, the activities of aminopeptidase and endoprotease at acid or neutral pH increased until the 10th d of regrowth (Fig. 3A, C, D), while caboxypetidase was more stable during the 24 d of regrowth (Fig. 3B). The endoprotease activity showed an immediate increase after shoot removal, whereas aminopeptidase and carboxypeptidase activities showed an initial lag for the first 3 or 4 d of regrowth (Fig. 3). Although the interpretation of results comparing with other studies has been complicated by variation of plant materials, assay methods and the expression mode of result. We could be taken to conclude that the remobilization of soluble protein is associated with the increase of proteolytic activities. In a senescing leaves of cereal crops, the catabolism of soluble proteins was accompanied by the rapid decrease of aminopeptidase, while carboxypeptidase activities remained stable for a longer time or slightly increased (Perez *et al.*, 1973; Cheng and Kao, 1984). Endoprotease at acid or neutral pH increased generally (Feller and Erismann, 1978).

The data obtained in this experiment showed that total protein contents in roots significantly decreased from 36.33 to 21.21 mg protein·plant$^{-1}$ during the first 10 d of regrowth and rapidly recovered from d 11 to d 24, while those in regrowing leaves increased throughout the entire experimental period (Table 1). These results well coincide with the time of nitrogen remobilization from roots to regrowing shoots, which was previously determined using $^{15}N$ labeling (Kim *et al.*, 1991). On the other hand, total activities of endopeptidase in roots highly increased during the first 4 d, and then slightly decreased until d 10,

**Table 1.** Changes in total protein contents and total activities of proteolytic enzymes in regrowing shoots and roots during a regrowth period of 24 d. The values are expressed as mg protein or unit of enzyme activity per plant. Each value is the mean± SE of three replicates

| d of regrowth | Protein (mg) | Amino-peptidase | Carboxy-peptidase | Azocaseinase at pH 5 | Azocaseinase at pH 7.5 |
|---|---|---|---|---|---|
| | | Regrowing shoots | | | |
| 4 | 5.4± 1.1 | 11.8± 2.7 | 10.7 ± 0.9 | 1.8± 0.1 | – |
| 6 | 8.2± 1.4 | 15.3± 1.9 | 16.3 ± 2.9 | 2.5± 0.9 | – |
| 10 | 22.5± 3.6 | 36.8± 3.0 | 42.64± 3.4 | 5.8± 0.8 | – |
| 14 | 32.4± 5.3 | 47.4± 3.5 | 61.0 ± 5.1 | 7.6± 0.5 | – |
| 24 | 89.9± 9.8 | 134.8± 10.4 | 179.8 ± 9.8 | 23.4± 1.6 | – |
| | | Roots | | | |
| 0 | 36.3± 2.1 | 58.2± 4.1 | 154.4± 11.3 | 39.2± 1.9 | 7.3± 0.6 |
| 1 | 35.9± 1.4 | 55.4± 3.6 | 148.3± 9.9 | 63.9± 2.4 | 14.4± 0.9 |
| 2 | 32.2± 1.1 | 55.7± 3.8 | 142.1± 8.3 | 68.9± 3.5 | 15.5± 0.4 |
| 3 | 30.2± 0.7 | 57.3± 3.6 | 132.8± 9.2 | 75.3± 3.7 | 15.7± 0.5 |
| 4 | 29.9± 1.6 | 64.9± 5.4 | 155.9± 10.6 | 86.5± 4.4 | 17.6± 0.7 |
| 6 | 26.2± 1.3 | 82.6± 9.1 | 149.4± 11.4 | 82.4± 2.8 | 17.3± 0.8 |
| 10 | 21.2± 2.0 | 84.4± 5.8 | 115.4± 8.1 | 78.3± 2.2 | 15.9± 0.5 |
| 14 | 27.7± 1.2 | 99.3± 4.8 | 123.4± 6.7 | 88.6± 5.1 | 18.8± 1.0 |
| 24 | 51.4± 3.3 | 138.1± 6.6 | 272.9± 14.6 | 154.2± 10.6 | 30.8± 2.6 |

while those of aminopeptidase and carboxypeptidase were not significantly influenced by shoot removal during the first 4 d. The activities of all enzyme examined rapidly increased from d 11 to d 24 (Table 1). These results indicate that the degradation of roots protein actively occurs during the first 4 to 6 d, and it slightly decreases until d 10 when protein reserves are already lowed. The rapid increase of total activities from d 11 to d 24 may possibly associated with the inflow of newly assimilated nitrogen from the medium as suggested in a previous study (Kim *et al.*, 1991). Considering the characteristics of enzyme activities and protein remobilization in terms of sink/source relationship, it is possible to draw some general conclusions. The sink organ, regrowing leaves in this study, could be distinguished by the increase of total protein content and total protease activities, while protein contents remained stable after shoot removal. The source organ, roots in this study, is characterized by the increase of endoprotease activities and the decrease in protein contents and in its total content during the period of active protein remobilization.

We, therefore, suggest that the roots of shoot-removed plant exhibit source behavior for proteins during the early regrowth, and that endoprotease plays an important role in protein degradation. However, the activities of aminopeptidase and carboxypeptidase are not consistently coincided with the time of root protein degradation.

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# 알팔파(*Medicago sativa*)의 刈取 後 再生時 蛋白質 含量 및 蛋白質 分解酵素 活性의 變化

金 太 桓* · 金 炳 鎬[1]
慶尙大學校 農科大學 畜産振興硏究所, [1]酪農學科

## 적 요

다년생 사료작물인 알팔파(*Medicago sativa* L.)의 재생시 단백질 대사를 규명하기 위해 수경재배하여 개화 초기에 지상부위를 예취한 후 재생 24일 동안의 잎과 뿌리의 단백질 함량 및 단백질 분해효소 활성을 각각 분석하였다. 재생 초기 10일 동안 잎과 줄기의 재생은 매우 완만하게 진행되다가 재생 11일부터 건물 함량이 현저히 증가하였다. 재생기간중 단백질 함량은 잎의 경우 예취에 의한 유의적인 변화를 보이지 않았으나, 뿌리의 경우 예취 후 재생 초기 10일간 약 36%의 감소를 보였다가 재생 11일부터 점차 회복되었다. 재생기간중 잎에서의 aminopeptidase와 endoprotease 활성은 약간 감소하는 경향을 나타내었다. 뿌리에서의 endoprotease 활성은 예취 후 초기 10일 동안 급격히 증가하였다가 이후 안정되었는데 재생 10일차의 활성은 예취일 수준의 370%로 가장 높았다. 이상의 결과들로부터 알팔파의 예취 후 초기 10일 동안 뿌리내 단백질의 활발한 분해가 일어나면 endoprotease 활성이 이에 밀접하게 관여함을 알 수 있었다.

주요어 : 알팔파, 재생, 단백질 함량, 단백질 분해효소 활성

*교신저자 : Fax (0591) 751-6113